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Thesis

THE DETERMINATION OF QUINIDINE IN BLOOD

by

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INTRODUCTION

Quinidine, which is the optical isomer of quinine, is used in the form of its sulfate as a cardiac depressant. Quinidine sulfate has been used for many years to combat exaggerated excitability of the heart muscle in the therapeutic treatment of auricular fibrillation, tachycardia and extrasystoles. As early as 1914, the effect of cinchona alkaloids on heart action was noted by Wenchebach[13] and in 1918 Frey[13] demonstrated that of all the cinchona alkaloids quinidine was the most effective. Since that time there has been a steady stream of papers describing the action and effects of quinidine and other cinchona alkaloids on cardiac rhythm. However, the early indiscriminate use of quinidine resulted in it being branded as a dangerous drug due to its toxicity. Disuse followed, and it is only in recent times and with the study of the toxicity of quinidine that it once more has come into general use. The exact mechanism of the operation of quinidine in reestablishing cardiac rhythm has not yet been conclusively shown. The consensus seems to be that quinidine prolongs the refractory period of the heart muscle and also reduces the excitability of the heart. It has even been suggested by de Boer and Holtkamp[2] that the therapeutic action of quinidine is due to hydroquinidine which is present in small amounts as an impurity in preparations of quinidine.

The study of a drug must be divided into two distinct aspects. The first, obviously, is the therapeutic effect which will be demonstrated on the administration of the drug. The second phase of the study, and perhaps the most important, is the physiological disposition of the drug in the body. To facilitate the study of the absorption, distribution, degradation and excretion of quinidine it was necessary to devise analytical methods to

determine the amount of the drug present in body tissues and body fluids. The medium of exchange of a drug in the body is the blood stream. From the measurement of quinidine in blood or plasma an indication of its disposition in the body can be obtained. Many methods to determine quinidine in body fluids and body tissue have been described. Most of these procedures are strictly research methods which are so laborious or require such unusual equipment that they are impractical as far as the ordinary laboratory is concerned.

Recent findings indicate that high plasma levels of quinidine are extremely dangerous. Death, preceded by indications of ventricular fibrillation, has been induced in a group of dogs[20] when the quinidine plasma levels were in the range of 10 to 15 milligrams per liter of plasma. Patients supposedly having died of ventricular fibrillation who had high post-mortem quinidine plasma levels in this range have cast strong suspicion on quinidine as the actual cause of death.[20] In view of these findings and with the increased use of quinidine, it is believed that quinidine blood levels should be run on all patients undergoing extended quinidine therapy. If this procedure is followed, the danger level can be seen and the therapy stopped before dire consequences result.

It is the purpose of this research to investigate the existing analytical methods of measuring quinidine blood levels; to make modifications and changes and to present a method to the average laboratory which will enable quinidine blood levels to be run easily, quickly and with the materials and equipment available in the majority of hospital laboratories. The method should be selective and sensitive enough to present a good picture of quinidine blood levels which in conjunction with clinical study will result in the safe and effective use of quinidine.

HISTORICAL REVIEW

The strictly chemical methods that have been described to determine alkaloids in body fluids can be divided in four major groups. These divisions are (1) gravimetric and volumetric, (2)nephelometric, (3) fluorometric and (4) colorimetric. Although my work concerns the determination of quinidine, since quinidine and quinine are so similar in chemical nature and many of the quinine methods can be applied to quinidine, a description of methods for other alkaloids particularly quinine has been included.

Gravimetric and Volumetric Methods-A few methods employing these analytical procedures have been proposed, but they are comparatively insensitive and they are all extremely laborious. These methods can only measure large quantities of alkaloid and are not applicable to the measurement of quinidine in blood where the quinidine is found in very small amounts.

Monnet[22] described both a gravimetric method and a volumetric method. In the gravimetric method, the quinidine is precipitated and weighed as a salt of HCNS. This salt is soluble to an extent of one part in 1477 at 20°C. His volumetric method employs the same idea, but in this case a definite excess of 0.1 normal KCNS solution is added and the excess KCNS remaining after the quinidine present is precipitated is titrated with 0.1 normal silver nitrate. The difference between the amount added and the amount available for titration is equivalent to the quinidine present.

Thomis[31] has described a method in which quinine is extracted in alkaline chloroform, and the separated chloroform solution titrated directly with 0.1 normal alcoholic hydrochloric acid using a microburet. The indicator is two drops of 0.2 per cent bromophenol blue. Thomis claims that the indicator will change color abruptly from violet to yellow on the

addition of 0.02 ml. of acid. He lists other alkaloids and the indicators that can be used in their titration, but quinidine is not included among them.

In the procedure proposed by O. Efinemko[8], the alkaloids are transferred from the ether-chloroform extracting solvent to a solution of 1 per cent hydrochloric acid by dissolving the remaining residue after evaporation in the acid. The acid solution is titrated with bromine water which has previously been standardized against a known pure quinidine hydrochloric acid solution. Rhodamine is used as the indicator.

Nephelometric Methods-Several methods involving nephelometry appear in the literature. The principle of nephelometric methods involves the formation of a turbidity with silicotungstic acid, and then comparing the turbidity with standards. However, Kyker and Lewis[18] who have described such a method for alkaloids point out that these methods cannot be used to determine quinidine since the silicotungstate formed with quinidine settles out too rapidly to be measured. The earliest methods of measuring quinine made use of this principle. Acton and King[1] described a turbidimetric method in 1921 and Kondi and Foy[11] also described such a method in 1936. These methods involve extracting the quinine in ether, evaporating the solvent and taking up the residue in 0.5 normal hydrochloric acid. Silicotungstic acid is added to form a turbidity which is compared with standards in a Pulfrich photometer using special standardized turbidity prisms. Kyker and Webb[19] modified the analytical method of Vedder and Masen[33] and incorporated the measurement of the turbidity in an Evelyn Photometer. The authors claim that this is the best method for determining quinine in blood. Marshall and Rogers[21] investigated this method and report that it is too insensitive

for general application.

Fluorometric Methods-Fluorometric methods to determine the alkaloid content of body fluids are based on the property of certain organic compounds to fluoresce when they are exposed to ultraviolet light. Quinidine possesses this property and probably fluorometric methods are the most sensitive means of measuring quinidine in blood. In 1925 Rene Fabre[10] noted the fact that the fluorometric intensity of a substance did not vary directly with its concentration in solution, but reached a maximum at a certain determinable concentration. Using this principle he was able to determine the concentration of fluorescent material present in a solution.

An early method for determining quinine in blood was described by Pantschenkow and Kustner[23]. They dissolved the ether extract of whole blood in sulfuric acid and compared its fluorescence in a Bachschen quartz lamp with the fluorescence of quinine bisulfate standards.

Kaiser[16] has described a similar method in which whole blood is treated with sodium hydroxide and sodium citrate, heated to 80°C., and the alkaloid extracted from this mixture with chloroform. The chloroform solution is evaporated to dryness and the residue extracted with an ether 0.1 normal sulfuric acid solution. The residue of the extraction is exposed to ultraviolet light and its fluorescence compared with standards.

O. M. Efinemko[9] modified his previously described volumetric method into a procedure which involves both fluorometry and a titration procedure. He extracted the alkaloid into a chloroform-ethyl ether solution and treated the mixture with bromine water. As an indicator he used the quenching of the fluorescence in the light of a Hannan quartz lamp. He

stated that he used bromine water rather than iodine in his titration because bromine forms a soluble compound with alkaloids while the iodides are insoluble.

The first method employing fluorometry that gave good recoveries was published in a paper by Kelsey and Geiling[17] in 1942. They state that the fluorescence of a sulfuric acid solution of quinine is the most sensitive determinative method for this alkaloid and that they were able to measure as little as 0.5 micrograms of quinine. Their method involves the extraction of the quinine into a 0.1 normal sulfuric acid solution and comparing its fluorescence against the fluorescence of a known standard using the Coleman Universal Spectrophotometer.

Perhaps no one has done more experimental work in devising methods to determine cinchona alkaloids in body fluids than Bernard B. Brodie[3,4,5] and his associates. Among his many methods is included a fluorometric[5] method which is perhaps the quickest way of determining blood alkaloid levels since it does not involve an extracting process. A protein precipitate is made with meta phosphoric acid and the fluorescence of the filtrate measured in the Coleman Fluorophotometer. This fluorescence is compared with standards prepared with plasma filtrates as the aqueous phase and known amounts of alkaloid. The reason for using the plasma filtrates in preparing standards is given as being due to the presence of substances in plasma filtrates which quench fluorescence. The authors note that quinidine is the only important cinchona alkaloid other than quinine which fluoresces appreciably and for this reason this method can be applied to the determination ofquinidine in blood.

The latest fluorometric method described was proposed by Unti[32] in

1944. However, this appears to be much more laborious than either the method of Kelsey and Geiling[17] or that of Brodie[5] although Unti claims that he was able to recover 0.02 milligram per cent quinine from blood. The blood is dehydrated with anhydrous sodium sulfate, the solids moistened with a few drops of concentrated ammonium hydroxide, and the alkaloids extracted three times with varying mixtures of a chloroform ethyl ether mixture at a temperature of 65°C. The combined extracts are evaporated to dryness and the residue dissolved in 2 percent sulfuric acid. The fluorescence of this mixture is then measured and compared with the fluorescence of known standards.

Colorimetric Methods-Colorimetric methods are based on the property of alkaloids to form colored compounds or colored complexes with certain chemicals, the intensity of the color being proportional to the concentration of the alkaloid present. The methods to be described differ in the substances used to form the colors.

As early as 1931 Vedder and Masen[33] described a colorimetric method for the determination of alkaloids in blood. The alkaloid is extracted from blood with ether and the residue remaining after the ether is evaporated to dryness, taken up in 5 ml. of 2 normal sulfuric acid saturated with zinc sulfate. The color was formed by adding gum arabic solution and then a potassium bismuthous iodide reagent. Standards were prepared at the same time as the unknown and their colors compared in a colorimeter immediately. The reading must be made within two minutes of mixing since the reddish brown color changes and fades in two minutes. The authors claim that the sensitivity of the method is 2 mg. per liter of blood.

The most recent colorimetric methods for determining alkaloids are

all based on the ability of alkaloids to form colored complexes with acid dyes. Prudhomme[25] was the original worker to employ this method. He used a 2 per cent solution of eosin. Blood is treated with saturated sodium sulfate and normal sulfuric acid at a temperature of 45-50°C. The filtrate is then buffered at pH 7 and the eosin added. The red color which is formed is extracted in chloroform and compared colorimetrically with previously prepared standards. As later writers[21] have pointed out, the drawback to the use of eosin is the very faint color that is formed and the complex formed is too easily adsorbed to the sides of the container.

Brodie's[4] method based on a color complex of quinidine and methyl orange hasbeen used in clinical blood determinations to some extent. This method is described in detail in the experimental part of this thesis.

Marshall and Rogers[27] have recently claimed that bromothymol blue will yield an extractable color with alkaloids. The method will detect as little as 0.25 micrograms per milliliter.

A study of the literature shows that the only methods that are practical for determining quinidine in blood are the fluorometric and the colorimetric methods. Although the fluorometric methods are quicker and more sensitive, the colorimetric methods do not require expensive laboratory equipment and possess adequate sensitivity for clinical work.

EXPERIMENTAL PROCEDURE

After reviewing the various methods of analysis that have been devised to determine cinchona alkaloids in plasma and blood it was decided that the method described by Brodie[4] and his co-workers offered the greatest possibilities of being modified into a method suitable for the average laboratory.

This method is based on the fact that cinchona alkaloids and sulfonic acids join to form colored complexes of high molecular weight which are soluble in organic solvents. Since the amount of the sulfonic acid entering the reaction is directly proportional to the amount of alkaloid present, a measurement of the sulfonic acid complex present in the organic solvent is also a proportional measurement of the amount of alkaloid present. Methyl orange was the sulfonic acid chosen since it has a high color index and forms a complex with quinidine that is highly soluble in ethylene dichloride which is the organic solvent used.

The procedure for determining cinchonidine in plasma as described by Brodie[4] and his associates is quoted verbatim at this point since the experimental procedure to be described has made use of much of this protocol.

> "PROCEDURE FOR PLASMA-Add 1 to 5 ml. of plasma and 1 ml. of 1 N NaOH to 20 ml. of ethylene dichloride in a 60 ml. glass stoppered bottle and shake for 5 minutes preferably on a shaking apparatus. (NOTE: The pH of the aqueous phase during the initial extraction is not critical, provided it is higher than 9.) Decant the contents of the bottle into a 40 ml. round bottomed tube and centrifuge for 10 minutes at 2500 RPM to break the emulsion. Remove the supernatant aqueous layer by aspiration. (NOTE: A solid emulsion sometimes forms in the ethylene dichloride. This may be broken by vigorous stirring with a glass rod. A second centrifugation will then produce a clean separation of the two phases.) Return the ethylene dichloride solution

to a 60 ml. glass-stoppered bottle (the original thoroughly rinsed out bottle may be used), restraining any coagulum present with a stirring rod. Add an equal volume of the alcoholic KOH solution and shake for 10 minutes. (NOTE: This step serves to remove a blank from plasma. It is believed that this blank is caused by the absorption of methyl orange on small particles floating in the solvent and that this particulate matter is removed by the alcoholic KOH wash.) Transfer the contents to a 40 ml. tube (the original rinsed out tube may be used) and centrifuge for 1 minute at 2500 RPM. Remove this supernatant aqueous layer completely by aspiration and decant the ethylene dichloride into a 60 ml. glass stoppered bottle (the original thoroughly rinsed out bottle may be used.) (NOTE: It is important that all traces of alkali be removed, since any that remains will alter the pH of the methyl orange reagent added in the next step of the procedure. This constitutes a major source of error in the routine application of the procedure.) Add 0.5 ml. of methyl orange reagent and shake for 5 minutes at 3000 RPM. Carefully remove all the supernatant layer by aspiration, decant the ethylene dichloride phase into a 25 ml. test-tube and recentrifuge for 5 minutes. Pipette 10 ml. of the ethylene dichloride into a colorimeter tube containing 1 ml. of the alcoholic sulfuric acid and mix thoroughly. Read in the colorimeter with a filter having a maximal transmission at 540 mu.

A reagent blank in which water is substituted for plasma is run through the same procedure and is used for setting the instrument to 100 percent transmission. This reagent blank should not give a transmission of less than 97 when ethylene dichloride plus the alcoholic sulfuric acid is used to set the instrument to 100 (Evelyn photoelectric colorimeter)"

Preparation of Reagents[4]

1. Quinidine Standard-A solution of quinidine was made using quinidine sulfate dihydrate in 0.1 normal sulfuric acid. The stock quinidine solution was made at 100 mg. per liter as quinidine and more dilute solutions were made with distilled water. The stock quinidine solution was prepared by dissolving 120.7 mg. of quinidine sulfate dihydrate per liter.

2. Ethylene dichloride-A commercial grade (Eastman Kodak) of ethylene dichloride was used. To remove any extraneous organic material that

might be present in the ethylene dichloride and later interfere with the methyl orange reaction, purification was accomplished by first washing the ethylene dichloride with one fifth its volume of 1 normal hydrochloric acid and then after separating the two layers, washing the ethylene dichloride with distilled water. The two phases (both hydrochloric acid and the water washings) were allowed to remain in contact overnight before separation.

3. Sodium Hydroxide, 1 normal.

4. Alcoholic potassium hydroxide solution-0.1 normal potassium hydroxide in 20 per cent ethyl alcohol.

5. Alcoholic sulfuric acid solution-2 ml. of concentrated sulfuric acid in 100 ml. of absolute alcohol.

6. Methyl orange solution-The methyl orange solution was prepared as a saturated solution of the sodium salt of methyl orange in 0.5 molar boric acid. The saturated solution is prepared by adding an excess of the sodium salt of methyl orange to the boric acid solution, heating the mixture gently and then filtering after the solution has cooled to room temperature. According to Brodie 0.5 molar boric acid is used as the buffer to keep the pH at about 5 (optimum pH for the complex formation to take place) rather than a salt buffer because inorganic salts lower the solubility of methyl orange.

Apparatus-The only apparatus used was 50 ml. glass stoppered volumetric flasks in which all mixing was done, standard volumetric and graduated pipettes, a shaking apparatus, a centrifuge which was capable of speed up to 3000 RPM, 50 ml. centrifuge tubes, and an Evelyn Colorimeter using a filter with a maximum transmission of 540 mμ. There is no need to use

volumetric flasks and ordinary ungraded flasks can be used with equal results.

Method With Standards

The quantity of quinidine used was made by diluting the prepared quinidine sulfate solution. One ml. of this solution was added to a 50 ml. flask and diluted to 5 ml. with water. One ml. of 1 normal sodium hydroxide was added and 20 ml. of purified ethylene dichloride were entered into the flask from a burette. The mixture was shaken for five minutes on a shaking apparatus and then transferred to a 50 ml. centrifuge tube in which it was centrifuged for five minutes at a speed of about 3000 RPM. The aqueous layer which is on top is carefully removed by aspiration. Aspiration is accomplished by the use of a dropper with a capillary end. Near the interface the bottom layer in the dropper is returned to the original liquid and the upper layer is discarded. One ml. of water is added directly to the centrifuge tube and the mixture is stirred with a glass stirring rod. After centrifuging for five minutes at 3000 RPM the aqueous layer is again removed. The mixture is then returned to the previously used flask which has been thoroughly rinsed with water and a 0.5 ml. portion of the methyl orange solution is added. After shaking for five minutes the mixture is centrifuged for five minutes. An aliquot of 10 ml. is added to an Evelyn colorimeter tube containing 1 ml. of the alcoholic sulfuric acid solution. The color is measured in the Evelyn Photoelectric Colorimeter using a filter with a maximum transmission of 540mμ. The colorimeter is set to 100 per cent transmittance with a mixture of 10 ml. of ethylene dichloride plus 1 ml. of the alcoholic sulfuric acid solution.

Modifications in Present Method

Removal of alkali-In order to extract the quinidine from the aqueous layer into the ethylene dichloride, an alkaline pH is necessary. The distribution of alkaloid extracted at various pH has been described by Brodie[4] and is included in the appendix. The pH must be in the vicinity of pH 5 when the methyl orange solution is added to give optimum complex formation between the methyl orange and the quinidine present. Great care must be taken to remove the alkali before the methyl orange is added. If the pH remains too high, inaccuracies will result.

Brodie mentions that the alkali must be removed before the methyl orange is added, but he gives no practical way of doing this. Aspiration alone is not sufficient to remove all of the alkali present. We have found that the few drops of alkali that remain after aspiration are sufficient to make the solution alkaline enough to give erratic results. (page 26) If the pH is not in the vicinity of pH 5 varying amounts of the methyl orange complex will result. It was found that if a water wash is introduced the alkali will be sufficiently removed to insure good results. The original aqueous layer is removed as well as possible by aspiration and 1 ml. of water is added. The mixture is stirred well and after centrifugation, the aqueous layer is removed by aspiration. Experiments were tried using various amounts of water in the washings and it was found that the amount of water used in the water washings did not play a part in giving the desired results. Equal results were obtained with all the volumes that were tried. It was decided to use 1 ml. of water since this is an easily measured volume and the ratio between the organic phase and the aqueous phase is kept large.

Reagent Blank-Another modification made to Brodie's method concerned the use of a freshly prepared reagent blank to set the Evelyn Photoelectric Colorimeter to 100 per cent transmittance. The use of a reagent blank for this purpose is an unnecessary procedure since the reagents are quite stable and the value of a reagent blank obtained one day will not change from that obtained on the next, if the same stock of reagents is used. It was also found, using the ethylene dichloride alcoholic sulfuric acid mixture to set the instrument, that the value of the reagent blank was quite small. (Average absorbance value of 0.0093) Once an average reagent blank value has been obtained, the only use of the reagent blank is to verify the purity of new stocks of reagents as they are prepared. The reagent blank should be run after new solutions have been prepared and its value should fall in the range of the average reagent blank. In this way it can be determined if solutions have been purified properly.

It was decided that an ethylene dichloride alcoholic sulfuric acid solution made up in an Evelyn colorimeter tube as all samples, 10 ml. of ethylene dichloride and 1 ml. of the alcoholic sulfuric acid solution, could be used to set the photometer to 100 per cent transmittance. This was used as the blank to set the colorimeter in all the present work. It was also found that this blank remained stable when it was stoppered and kept in the refrigerator. Therefore a new blank was not prepared for each set of experiments, but the same one was used from day to day. Although the blank will probably remain constant for an indefinite period of time, a new blank was prepared every few weeks and checked against the old blank before the latter was discarded.

Removal of Methyl Orange-Brodie suggests that after the methyl orange

mixture has been centrifuged for the first time a second centrifuging is necessary after the supernatant layer of excess methyl orange has been removed and before the aliquot for Evelyn measurement is taken. The necessity for this second centrifugation was investigated and it was found that it was not needed, and did not improve the results. The same results were obtained with and without the second centrifugation. Therefore this was dispensed with and the mixture was centrifuged but once. It was also noticed that the supernatant methyl orange layer had a tendency to gather along the side of the glass centrifuge tube and leave the center area completely clear. If the pipette was inserted through this center area which is free of methyl orange, it was not necessary to remove all the supernatant methyl orange layer.

Effect of Time on Color-The question arose as to what was the stability of the methyl orange complex color formed and if time was a factor to be considered in making colorimeter readings. Experiments were carried out in which the colorimeter readings were continued over a period of time and even continued after the colored solution had stood over night. It was found that the color formed was very stable and that the colorimeter readings remained constant despite the time that elapsed between the time of preparing the aliquot and the colorimeter reading. In all experimental work the colorimeter readings were made as soon as possible after the aliquot was prepared.

Sensitivity-It wasshown that standard values taken using 0.5 mg. of quinidine per liter of solution did not follow the linearity of the other points. Therefore it can be assumed that values much below 1 mg. per liter of solution can not be determined in the present method.

Quinidine Standard Curves

The standard curves were prepared by determining the absorbance (optical density) of quantities of quinidine varying from 0 to 12 mg. per liter and plotting these values against the amount of quinidine. The optical density equals 2-log G, where G is the galvanometer reading or the per cent transmittance. In the equations of the standard curves, the value 0.0093 must be subtracted from the absorbance since it represents the absorbance of the average reagent blank.

Method With Plasma

4.5 ml. of plasma, 0.5 ml. of quinidine solution, 1 ml. of 1 normal sodium hydroxide, and 20 ml. of ethylene dichloride are added to a 50 ml. flask and shaken for five minutes. The mixture is poured into a centrifuge tube and centrifuged at full speed for five minutes. At this point a solid emulsion appears at the interface between the two phases and extends down into the ethylene dichloride phase. The procedure at this point is to remove the plasma-aqueous phase by aspiration down to the surface of the emulsion. 1 ml. of water is added to the mixture and stirred with a glass stirring rod until it is noticed that the emulsion particles become granular. After recentrifugation, the emulsion is usually broken sufficiently to allow an aliquot to be taken. This aliquot should be more than 15 ml. and was ordinarily 18 ml. The aliquot is taken by putting a finger over the top of a volumetric pipette and inserting the pipette through the remaining emulsion and then drawing the aliquot. The solid emulsion particles will be pushed aside and cling to the sides of the centrifuge tube. The aliquot is returned to the washed flask and an equal volume of alcoholic potassium hydroxide is added to the clear liquid.

After shaking for ten minutes, the mixture is centrifuged for five minutes. A second emulsion may be obtained at this point and the above procedure is followed to secure an aliquot. Care must be taken to see that the aliquot is more than 10 ml. in volume. If there is no emulsion, the alcoholic phase is removed by aspiration. The organic phase is then subjected to a water washing by mixing 1 ml. of water with the remaining ethylene dichloride. The aqueous phase is removed by aspiration and a 0.5 ml. portion of methyl orange is added to the organic phase in the rinsed flask. The solution is shaken for five minutes and centrifuged for five minutes at full speed. A 10 ml. aliquot is taken and is treated as in the method with standards.

Experiments with Plasma

Recoveries of quinidine from plasma (Table 5, page 26) were carried out in the same general manner as above. The quinidine was added to plasma which was obtained from normal individuals. Solutions of quinidine were prepared by diluting a standard solution made by dissolving quinidine sulfate dihydrate in water and storing the solution in the refrigerator. The refrigerated solution remained stable and was kept for several weeks before it was discarded.

Discussion of Plasma Method

Emulsions-Attempts were made to break the emulsions completely before the plan of taking aliquots was adopted. Both sodium acetate and sodium chloride in varying amounts were tried, but seemed to have little effect on the emulsions. Water alone seemed to work well in reducing the emulsion, but did not remove the emulsion completely. With each succeeding portion of water the emulsion seemed to be broken to a greater degree, but the

first portion of water and adequate stirring broke more of the emulsion than any following portion. Following one portion of water, the emulsion was broken sufficiently to allow an adequate aliquot to be taken.

Volume of Ethylene Dichloride-Since the final amount of ethylene dichloride used is only 10 ml., the fact that 20 ml. were used as the extracting solvent brought up the question as to whether a smaller amount of ethylene dichloride could be used. It was shown that the quinidine was extracted just as well into smaller amounts of solvent. However, it was found that the problem of emulsions and the necessity of taking aliquots made 20 ml. a convenient amount to be used to insure a final aliquot of 10 ml.

Extracting Agent-Brodie[5] states that benzene is an ideal extracting agent for certain alkaloids since it is specific to the alkaloid alone and it also makes the operation easier since the benzene floats on the aqueous phase and does away with the necessity of the various aspiration steps. Experiments were tried substituting benzene for the ethylene dichloride and it was found that the colors formed in the benzene were so faint in the comparatively large volumes used that they could not be measured. The alkaloid method described by Brodie[5] in which the color is finally extracted from the benzene into a small amount of acid solution offers possibilities of being modified into a method which will eliminate the plasma blank.

Plasma Blank-It was found that normal plasma contained a small amount of substances that form methyl orange complexes similar to the quinidine complex and give a blank reading which averaged the equivalent of about 0.5 mg. of quinidine per liter of plasma. This plasma blank varied from 0.17 mg. to 0.52 mg. quinidine per liter of plasma in different people.

Many attempts were made to modify the method to eliminate the normal plasma blank. However, none of these experiments gave completely satisfactory results and it was decided that the most feasible procedure would be to run a series of normal bloods and obtain an average blank which could be subtracted from all blood values. (Table 2, page 24) This blank value falls within the sensitivity of the method.

Experiments to Remove Blank

The effect of removing protein from plasma on the normal plasma blank was investigated in an attempt to eliminate the normal plasma blank. The use of a non-protein filtrate would also make the experimental procedure much easier since there would be no emulsions.

Trichloracetic acid filtrate-The first substance tried to remove the normal blank from both plasma and whole blood by precipitating the protein was a trichloracetic acid filtrate. As shown in Table A, page 24, using an equal volume of 10 percent trichloracetic acid and plasma, the blank value was actually increased (1.55 mg./liter vs. 0.30 mg./liter) and the recovery of added quinidine was incomplete. The filtrate was neutralized with 1 normal sodium hydroxide, After centrifugation, the supernatant fluid was filtered to remove a few floating particles. However it was noted in the literature[12] that quinidine occluded to filter paper. After confirming this finding (Table A, page 24), the filtration was subsequently not used. Recovery values (Page 24 were obtained using five volumes of six per cent trichloracetic acid, but the normal blank persisted. The fact that recoveries were made seems to dispute the reported finding that trichloracetic acid will precipitate quinidine from a solution.[28]

Tungstic Acid filtrate-The next filtrate that was tried was a tungstic

acid filtrate of both plasma and of whole blood. The procedure used was to add to one volume of plasma, three volumes of water, one-half volume of 10 per cent sodium tungstate, and one-half volume of 0.667 normal sulfuric acid. After centrifugation, the supernatant fluid was neutralized with 1 normal sodium hydroxide. Although the plasma blank was eliminated, the tungstic acid carried down the quinidine with the protein and no quinidine was recovered. (Table B, page 25)

Hydrochloric acid-heat-The protein of plasma was precipitated by heat and hydrochloric acid. A recovery was tried by adding quinidine in 1 ml. of water to 4 ml. of plasma plus 15 ml. of water. The mixture was heated to about 90°C. and 0.55 ml. of 0.25 normal hydrochloric acid was added gradually until the protein flocculated. An aliquot of 16 ml. was taken and neutralized with one drop of normal sodium hydroxide. Emulsions were experienced when the aliquot of filtrate and the ethylene dichloride were mixed. The blank was not removed, but an 83.4 percent recovery was obtained (Table C, page 25).

Meta phosphoric acid-Although Brodie used a metaphosphoric acid filtrate in his fluorometric method, metaphosphoric acid was not tried as a precipitating agent. The necessity of using fifty volumes of reagent to bring about aprecipitation of the protein and leave the alkaloid free in the filtrate made a metaphosphoric acid filtrate impractical because of the resulting large volume of filtrate.

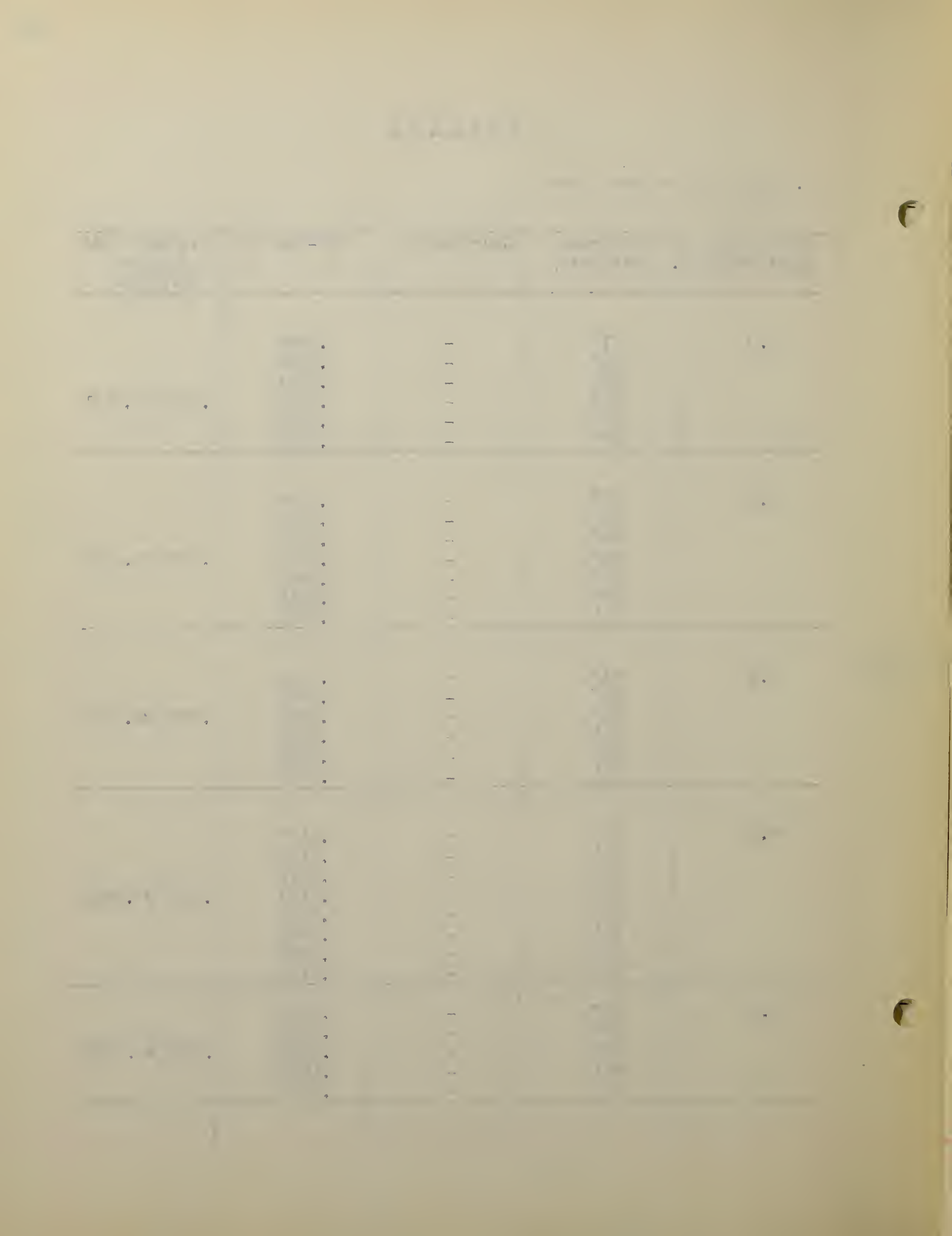
Whole Blood-Quinidine is present in both red cells and plasma.[15] In an attempt to neutralize the blank it was decided that if the quinidine could be extracted from the whole blood, the larger amount of quinidine extracted would make the quinidine blank ratio so small that the blank could be

discounted. Experiments were tried extracting the quinidine from hemolized blood. Although the recoveries are good (Table D, page 25) the problem of emulsions, and the fact that the blank is quite high prevent the adoption of a whole blood extraction to a clinical method. A sample of quinidine was added to 5 ml. of whole blood and the mixture allowed to stand for an hour to permit the quinidine to distribute itself between the cells and the plasma. Six volumes of water were then added and the mixture shaken. A 20 ml. aliquot of the mixture was taken for analysis. The very heavy emulsions that were formed required several water washings to break them sufficiently to allow aliquots to be taken.

RESULTS

1. Quinidine Standard Curve

Micrograms Quinidine/5 ml.	G (% Transmittance)	Corrected G	L(2-log G)	Average L and Standard Deviation
0.0	97^{3}	-	.0099	
	100°	-	.0000	
	98^{1}	-	.0077	
	96^{3}	-	.0144	.0093 ≠ .0051
	97^{1}	-	.0121	
	97^{1}	-	.0121	
5.0	87^{3}	-	.0568	
	88^{1}	-	.0543	
	89°	-	.0505	
	89°	-	.0505	.0550 ≠ .0041
	88^{3}	-	.0518	
	87°	-	.0617	
	87^{1}	-	.0593	
7.5	83°	-	.0809	
	85°	-	.0706	
	85^{3}	-	.0667	.0775 ≠ .0079
	84^{3}	-	.0718	
	82^{1}	-	.0862	
	81^{3}	-	.0875	
10.0	76^{1}	-	.1177	
	76^{1}	-	.1177	
	76^{1}	-	.1177	
	76^{2}	-	.1163	.1163 ≠ .0042
	75^{1}	-	.1235	
	76^{1}	-	.1177	
	77^{3}	-	.1093	
	77^{2}	-	.1107	
15.0	69°	-	.1612	
	69^{1}	-	.1596	
	69^{2}	-	.1580	.1581 ≠ .0058
	68^{2}	-	.1643	
	71^{1}	-	.1472	



Quinidine Standard Curve (Continued)

Micrograms Quinidine/5ml.	G (% Transmittance	Corrected G	L (2-log G)	Average L and Standard Deviation
20.0	64^{3}	-	.1888	
	63^{2}	-	.1973	
	66^{1}	-	.1788	.1947 ≠ .0113
	64^{3}	-	.1888	
	62^{3}	63^{0}	.2007	
	60^{3}	61^{1}	.2147	
25.0	52^{0}	52^{2}	.2798	
	52^{0}	52^{2}	.2798	.2830 ≠ .0050
	52^{1}	52^{3}	.2819	
	50^{3}	51^{1}	.2903	
30.0	44^{1}	44^{3}	.3490	
	43^{3}	44^{1}	.3540	.3550 ≠ .0048
	43^{1}	43^{3}	.3590	
	43^{1}	43^{3}	.3590	
40.0	35^{1}	35^{3}	.4470	
	34^{1}	34^{3}	.4590	.4625 ≠ .0120
	33^{1}	33^{3}	.4720	
	33^{1}	33^{3}	.4720	
50.0	26^{2}	27^{0}	.5690	
	25^{1}	25^{3}	.5890	.5738 ≠ .0111
	26^{3}	27^{1}	.5650	
	26^{3}	27^{1}	.5650	
60.0	20^{3}	21^{1}	.6730	
	20^{1}	20^{3}	.6830	.6805 ≠ .0050
	20^{1}	20^{3}	.6830	
	20^{1}	20^{3}	.6830	

The average slope of these points shows that there is a break in the plotted curves between the values of 20 micrograms of quinidine and 25 micrograms. There is linearity between 0 and 20 micrograms and between 25 and 60 micrograms. Therefore there are included in the appendix of this work three

graphs of these points. One represents the values between 0 and 20 micrograms.. The second, values between 25 and 60 micrograms, and the third graph is a composite curve derived by using the average slope of all the points. The curves were drawn by making use of the average slope of the experimental points.

2. Blank Values of Normal Plasma (all values corrected to 5.0 ml. plasma)

Ml. plasma	G (% Transmittance)	Corrected G	L (2-log G)	Value of Blank micrograms/5ml.
5.0	93^3	-	.0281	2.00
3.5	93^3	-	.0281	2.57
2.0	95^0	-	.0223	(not used)
4.0	$96'$	-	.0166	1.09
5.0	$96'$	-	.0166	0.86
6.0	$95'$	-	.0212	1.03

3. Experiments To Remove Plasma Blank

A. Recovery from Trichloracetic Acid Filtrate

Quinidine (micrograms)	G	L	Total recovery (micrograms)	G Blank	L Blank	Value Blank (micrograms)	Recovery (micrograms)	% Recovery
*50	$80'$	.0955	25.20	$91'$	.0398	7.73	17.5	35.2
*50	$81'$	.0901	23.50	$91'$	.0398	7.73	16.0	32.0
*50	70^3	.1503	27.00	$91'$	.0398	7.73	19.2	38.5
16	$66'$	.1708	31.80	$91'$	.0398	7.73	24.1	151.0
16	$72'$	.1412	24.73	$91'$	.0398	7.73	17.0	106.0

* Filtered through filter paper

B. Recovery from Tungstic Acid Filtrate

Quinidine (micrograms)	G (% Transmittance)	L (2-log G) -	Recovery (micrograms	% Recovery
0	100°	.0000	0	0
0	100°	.0000	0	0
5	99³	.0011	0	0
20	94¹	.0257	0	0

C. Recovery from Hydrochloric Acid-Heat Filtrate

Quinidine (micrograms)	G (% Transmittance)	L (2-log G	Recovery (micrograms)	% Recovery
0	95¹	.0212	1.55	-
20	70³	.1503	16.80	83.4

D. Recovery from Whole Blood (hemolyzed)

Quinidine (micrograms)	Volumes of Water	G	L	G (Blank)	L (Blank)	Recovery Total (micrograms)	Value Blank	Recovery (micrograms)	% Recovery
16	6	79°	.1024	94³	.0235	17.46	2.80	14.66	91.7
16	6	77³	.1093	94³	.0235	18.73	2.80	15.93	99.7
8	4	78³	.1037	87³	.0568	12.30	6.20	6.10	76.4
8	4	78³	.1037	87³	.0568	12.30	6.20	6.10	76.4

4. Necessity of Water Washing To Remove Alkali

In each case 10 micrograms of quinidine is used

Ml. of Water	G (% Transmittance	L(2-log G)	Average Standard L Value	Deviation (%) From Standard
0	86°	.0655	.1163	42.5
0	82'	.0848	.1163	27.2
0	85°	.0706	.1163	39.5
0.75	76'	.1177	.1163	1.2
0.75	76'	.1177	.1163	1.2
1.00	77'	.1121	.1163	3.6
1.50	76'	.1177	.1163	1.2
1.50	77[2]	.1163	.1163	0.0
3.00	75'	.1235	.1163	6.1

5. Recovery of quinidine added to plasma

a. Recoveries using an average plasma blank of 1.51 micrograms per 5 ml. of plasma.

Quinidine (micrograms)	G(% Transmittance	Corrected G	L(2-logG)	Total Recovery Value	Recovery (micrograms)	% Recovery
5.0	83'	-	.0796	7.30	5.79	115.3
5.0	83'	-	.0796	7.30	5.79	115.3
10.0	73[2]	-	.1332	13.00	11.49	114.9
10.0	75°	-	.1221	11.75	10.24	102.4
20.0	58'	58[2]	.2328	23.10	21.49	107.0
20.0	56'	56[2]	.2480	24.75	23.24	116.1
50.0	27'	27[3]	.5570	48.50	46.99	96.1

b. Recoveries using blank value determined from same blood.

Quinidine (micrograms)	Total Recovery Value	Blank (micrograms quinidine)	Recovery (micrograms)	% Recovery
5.0	7.30	2.57	4.73	94.6
5.0	7.30	2.57	4.73	94.6
10.0	13.00	2.00	11.00	110.0
10.0	11.75	2.00	9.75	97.5
20.0	23.10	3.08	20.02	100.1
20.0	24.75	3.08	21.67	108.1
50.0	48.50	1.09	47.41	95.0

6. Clinical Blood Values

a. Mr. M. - Fibrillator put on therapeutic dose on Friday night, converted Saturday morning. Blood taken on Monday, two hours after prophylactic quinidine dose of three grains. Dosage was three grains three times a day.

G (% Transmittance) - - - 83^2
L (2-log G) - - - - - - - - .0783
Total Recovery Value - - 7.18 micrograms
Average Blank Value - - - 1.51 micrograms

Recovery is 5.67 micrograms, plasma level is 1.13 mg./liter.

b. Mary F. - Intervertebral disk, demerol administered at 2:30 P. M. blood sample taken one hour later.

G (% Transmittance - - - 89^3
L (2-log G) - - - - - - - .0470
Recovery Value Equivalent
to Quinidine - - - - - - 3.92 micrograms
Average Blank Value - - 1.51 micrograms

This recovery shows that other drugs can interfere with quinidine levels. In this case the demerol is equivalent to a quinidine plasma level of 0.48 mg./liter.

c. Margaret S. - On prophylactic quinidine dose for months, blood taken at 11:00 A. M., two hours after administration of quinidine. Dosage was three grains twice a day.

G (% Transmittance)- 88^3
L (2-log G) - - - - .0518
Total Recovery Value-4.41 micrograms
Average Blank Value -1.51 micrograms

Recovery is 2.90 micrograms, plasma level is 0.58 mg./liter.

d Mr. K. - On prophylactic dose of quinidine.

G (% Transmittance) - 83 '
L (2-log G) - - - - .0796
Total Recovery Value- 7.32 micrograms
Average Blank Value - 1.51 micrograms

Recovery is 5.81 micrograms, plasma level is 1.16 mg./liter.

Clinical Blood Values (Continued)

e. Samuel T. - On prophylactic quinidine dose of three grains four times per day. A high plasma level was found and blood was taken one day later to confirm the high level. Part of the blood taken on the second day subjected to whole blood analysis (hemolysis).

G (% Transmittance)	L (2-log G)	Total Recovery (micrograms)	Average Blank (micrograms)	Recovery (micrograms)	Plasma Level (mg./liter)
59^2 corrected to 59^3	.2236	22.31	1.51	20.80	4.20
61^2 corrected to 61^3	.2093	20.82	1.51	19.30	3.86

By Hemolysis From Whole Blood

G (% Transmittance) - - - 62^3 corrected to 63^2
L (2-log G) - - - - - - .2007
Total Recovery Value 24.90 micrograms
Average Blank - - - - - 4.50 micrograms
Recovery - - - - - - - - 20.40 micrograms
Whole Blood Level - - - 4.08 mg./liter

A P P E N D I X

1. Distribution of Cinchonidine and Apparent Cinchonidine Between Water and Ethylene Dichloride at Various pH Values[4].

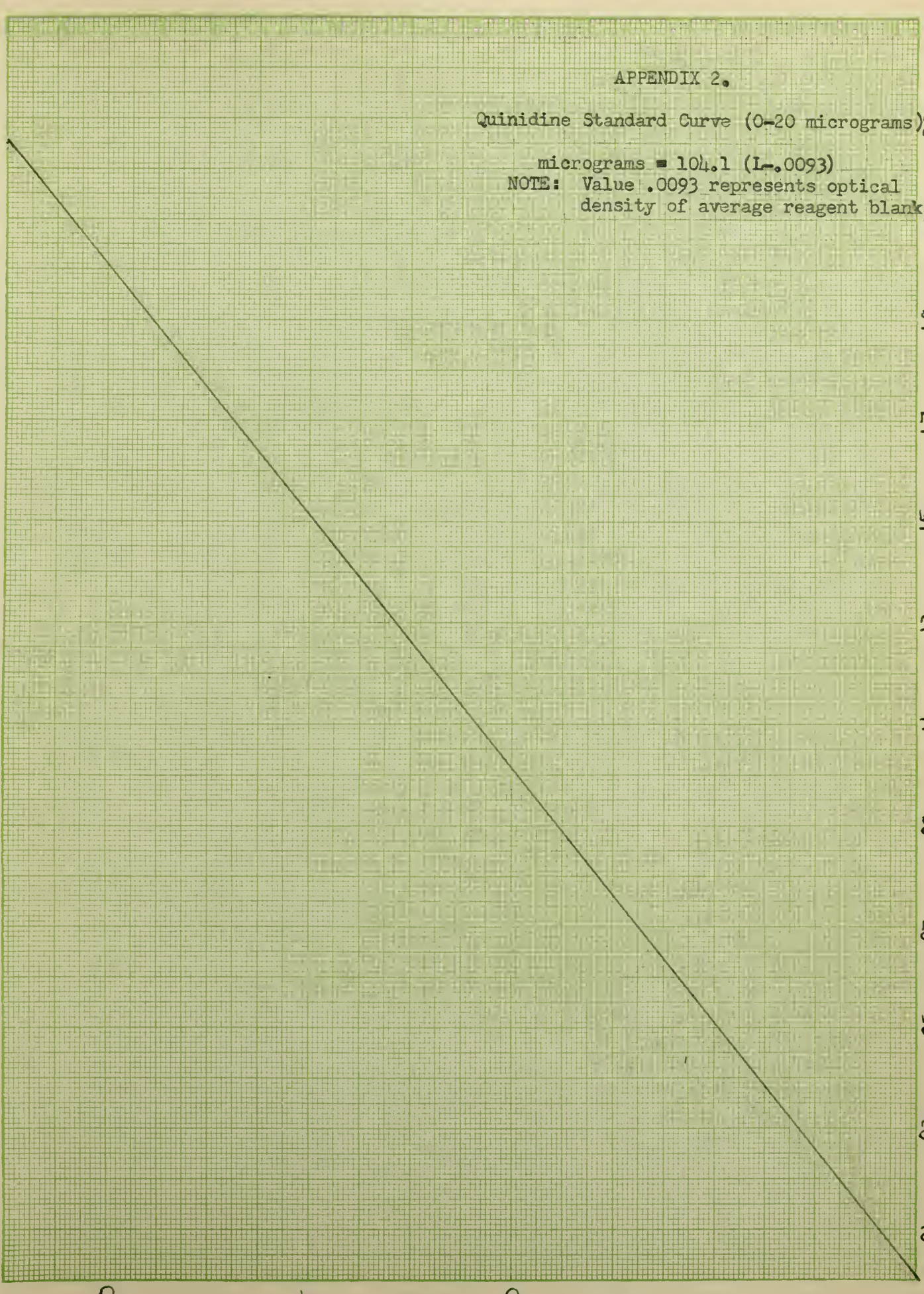
pH	ratio of concentration of drug in organic phase to total drug after shaking with appropriate buffer
5	0.02
6	0.15
7	0.64
8	0.92
9	0.97
10	0.98
11	0.97
12	0.99
13	1.00
14	0.99

APPENDIX 2.

Quinidine Standard Curve (0-20 micrograms)

micrograms = 104.1 (L-.0093)

NOTE: Value .0093 represents optical density of average reagent blank

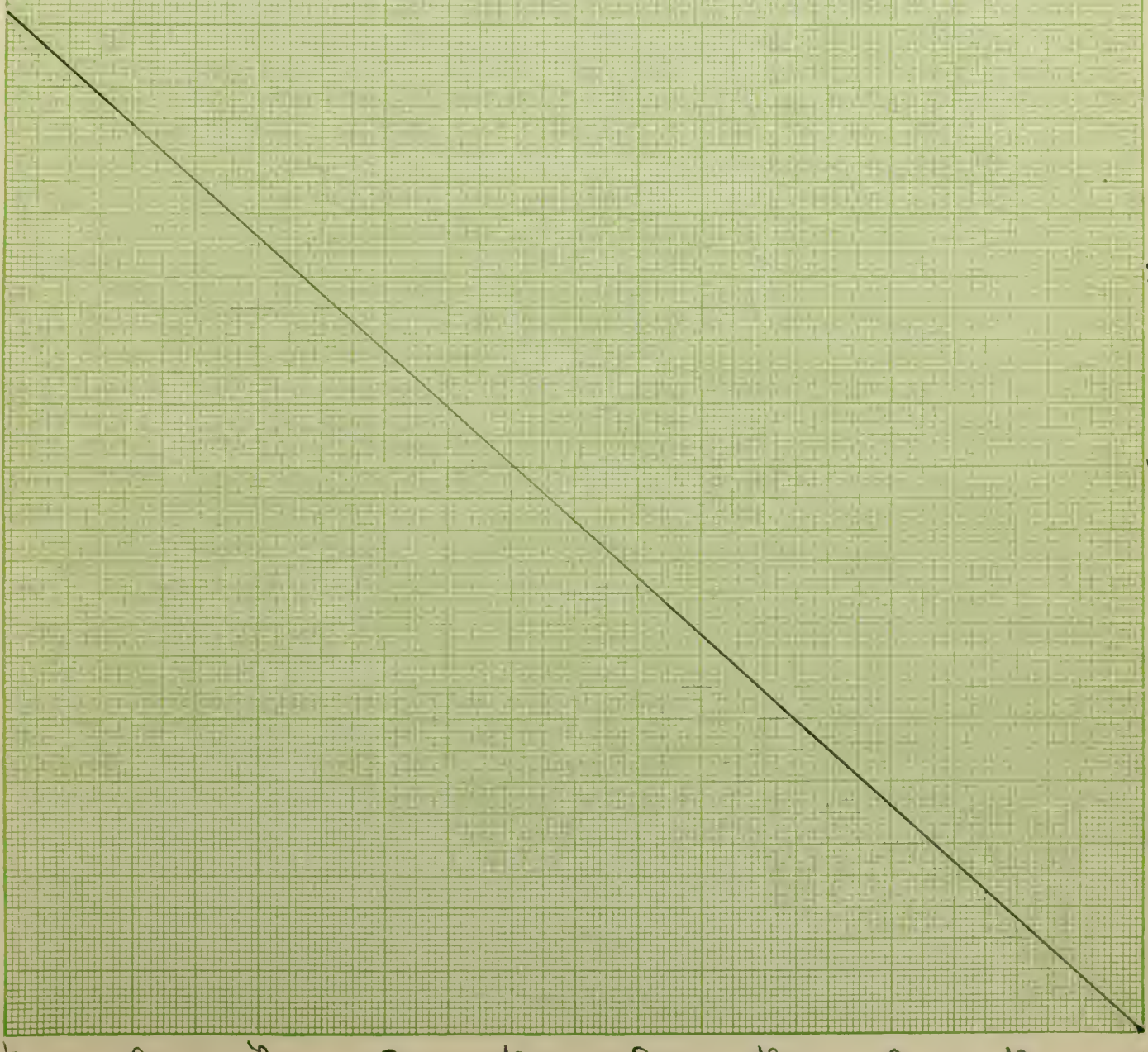


APPENDIX 3.

Quinidine Standard Curve (25-60 micrograms)

micrograms = 88.9 (L - .0093)

NOTE: Value .0093 represents optical density of average reagent blank.

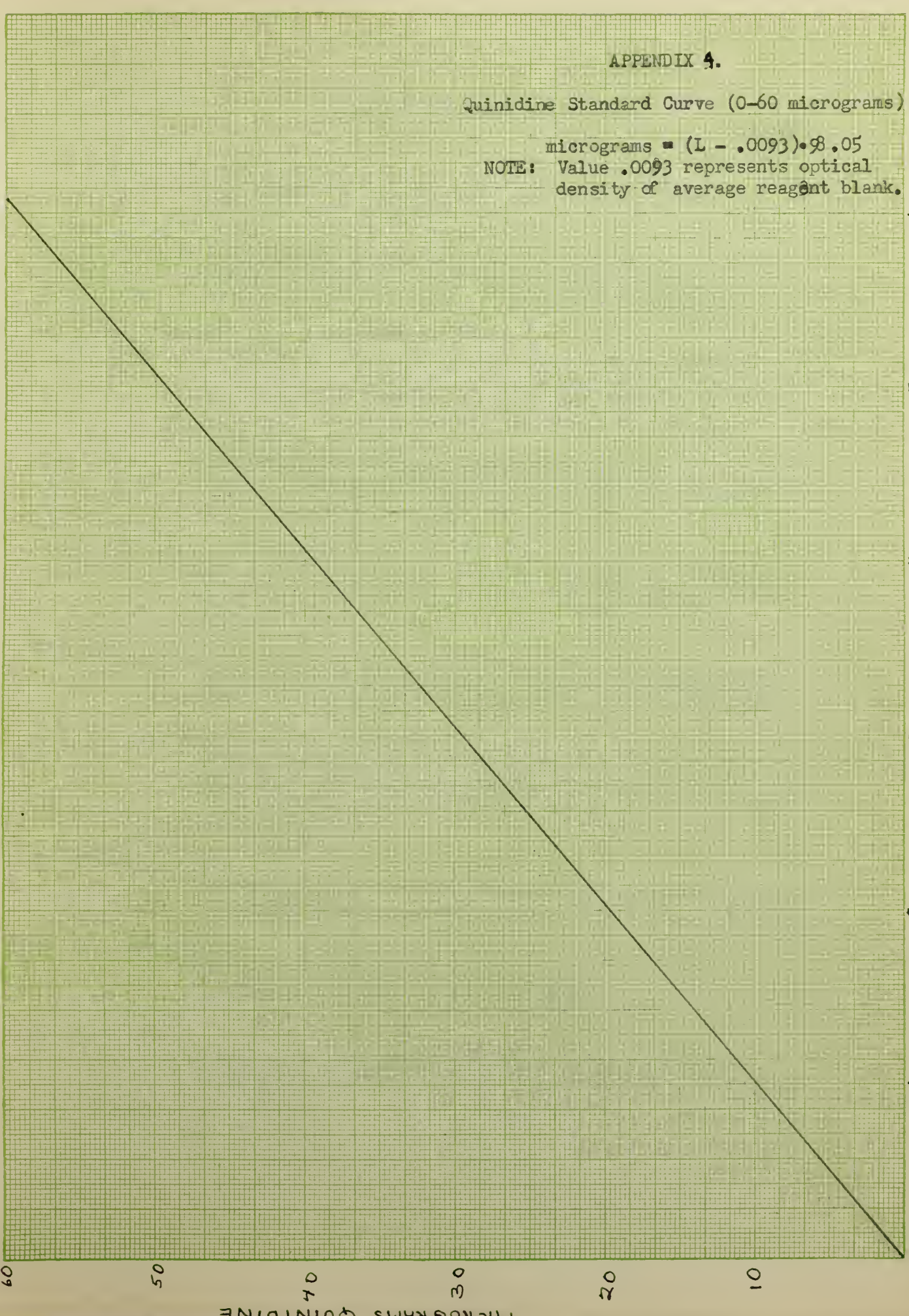


APPENDIX 4.

Quinidine Standard Curve (0-60 micrograms)

micrograms = (L - .0093)•98.05

NOTE: Value .0093 represents optical density of average reagent blank.



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SUMMARY

Brodie's[4] method for the colorimetric determination of alkaloids in blood, based on the formation of a color complex of the alkaloid with methyl orange, has been adapted to the determination of quinidine in blood. Quinidine in alkaline solution is extracted with ethylene dichloride, the extract washed with water and the washed extract treated with methyl orange. The violet color of the methyl orange-quinidine complex is developed in acid alcohol ethylene dichloride solution and measured photometrically. The principal modifications were made to take care of emulsions, to remove the alkali before the methyl orange complex is formed and in the method used to set the photometer to 100 per cent transmittance. The possibility of removing the normal plasma blank was also investigated.

The problem of emulsions which form between the plasma and the ethylene dichloride layers was overcome by taking aliquots from the emulsion containing solution. Enough of the emulsion is broken to allow a sufficient amount of clear liquid to be drawn off. The emulsion is partly broken by adding water to the emulsion mixture and stirring.

Since the pH of the solution must be alkaline to extract quinidine into solution, but then must be kept at pH 5 when the methyl orange complex is being formed, a practical means had to be devised to remove the alkaline layer completely before adding the buffered methyl orange solution. This cannot be done by aspiration alone and a water washing is introduced after aspiration to remove the remaining and interfering alkali.

The method of setting the photometer to 100 per cent transmittance was modified to make use of an ethylene dichloride alcoholic sulfuric acid

mixture for this purpose rather than using a freshly prepared reagent blank.

A drawback of the methyl orange method of plasma analysis is the fact that normally occurring substances in plasma produce a methyl orange complex color which results in a normal plasma blank reading. In the attempts to remove the blank various protein filtrates (trichloracetic acid, tungstic acid, and hydrochloric acid-heat methods) were used. It was finally decided that since the blank is small, if the average of a number of normal plasma was obtained this could be used as an average plasma blank and would fall within the sensitivity of the method. Experiments were also tried to remove the blank from whole blood. It was felt that since the whole blood contains more quinidine than plasma alone, the quinidine recovered/ blank ratio would be made much larger. However, this didnot prove to be so.

The above described method of determining plasma levels of quinidine is quick, simple and accurate enough to present a clear picture of the desirability of continuing quinidine therapy over a period of time. If a patient must be kept on such therapy, regular analyses will report increasing plasma levels before the danger level is reached.